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EFFECTS OF PHOSPHATE-MODIFIED ADENINE NUCLEOTIDE ANALOGUES ON INSULIN SECRETION FROM PERFUSED RAT PANCREAS

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1 The effects of three methylene analogues of adenosine 5′-triphosphate (ATP) or 5′-diphosphate (ADP) have been studied on insulin secrètion from the isolated perfused pancreas of the rat: 5′-adenylmethylene diposphonate or β,γ-methylene ATP, adenosine 5′-α,β-methylene triphosphate or α,β-methylene ATP and adenosine 5′-α,β-methylene diphosphonate or α,β-methylene ADP.

2 β,γ-Methylene ATP did not elicit any increase of insulin release; α,β-methylene ATP and α,β-methylene ADP induced a biphasic stimulation of insulin secretion; this effect was dose-related between 1.65 and 165 μmol/l. Relative potency ATP/α,β-methylene ATP was 1.2 and ATP/α,β-methylene ADP was 0.31.

3 Our results point to the importance of the steric and electronic characteristics of the polyphosphate chain of the analogues of ATP and ADP in inducing an insulin secretory effect. They support the hypothesis of a purine receptor for ATP and ADP.

Introduction

The insulin releasing effect of various adenine nucleotides has been established *in vitro* (Loubatières, Loubatières-Mariani & Chapal, 1972; Feldman & Jackson, 1974; Loubatières-Mariani, Chapal, Lignon & Valette, 1979). In this last paper the structure-activity study has allowed us to show that adenosine 5′-triphosphate (ATP) was the most potent of the nucleotides tested and that certain structural features were essential to elicit an insulin secretory effect: purine basis and di or triphosphate groups. Generally adenine derivatives are rapidly metabolized by enzymes; it could therefore be asked whether hydrolysis of these compounds have a part in their stimulatory effect. Adenine nucleotide analogues more resistant to degradation than ATP and ADP had to be used. Adenylimido diphosphate (AMP-PNP), a non phosphorylating structural analogue of ATP, causes dose-related increases in insulin secretion but is less potent than ATP (Loubatières-Mariani *et al.*, 1979).

The present study describes the effects of three other adenine nucleotide analogues. In these agents one oxygen atom of the di or triphosphate moiety is replaced by a methylene residue. The P-C-P bond is extremely stable and is not hydrolyzed. In adenosine 5′-triphosphate (ATP) the replacement of the anhydride oxygen linking the β and γ phosphorus atoms gives 5′-adenylmethylene diphosphonate or β, γ-methylene ATP; the replacement of the second anhydride oxygen which links the α and β phosphorus atoms gives adenosine 5′-α,β-methylene triphosphate or α,β-methylene ATP. In adenosine 5′-diphosphate (ADP) the replacement of the oxygen atom between α and β phosphorous atoms gives adenosine-5′α,β-methylene diphosphonate or α, β-methylene ADP (Figure 1).

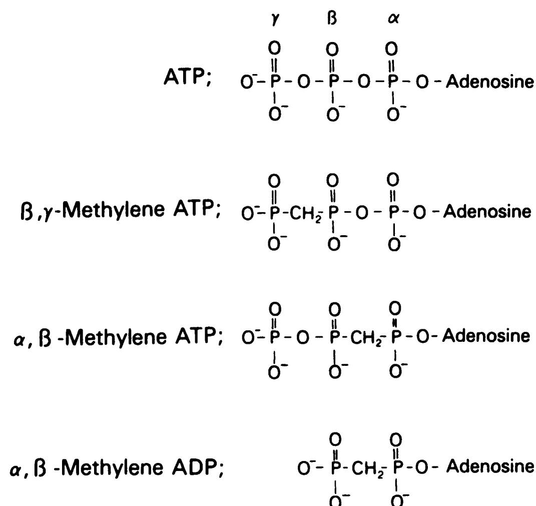

Figure 1 Structure of ATP and methylene analogues

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The aim of the present study was to determine the characteristic features in the polyphosphate chain needed for the stimulation of insulin secretion.

Methods

The experiments were carried out on the isolated perfused pancreas of the rat, according to the technique previously described (Loubatières, Mariani, De Malbosc, Ribes & Chapal, 1969). Male Wistar rats weighing 350 g and fed *ad libitum* were anaesthetized with 60 mg/kg sodium pentobarbitone by intraperitoneal injection. The pancreas was totally isolated from all neighbouring tissues and organs; it was perfused through its own arterial system with a Krebs-Ringer bicarbonate buffer containing bovine albumin (2 g/l). A mixture of O_2 (95%) and CO_2 (5%) was bubbled through this medium at atmospheric pressure. The pH of the solution was $\simeq$ 7.35. The preparation was maintained at 37.5°C. Each organ was perfused at a constant pressure and the flow rate was about 2.4 ml/min. The glucose concentration in the perfusion medium was 1.5 g/l (8.33 mmol/l).

In all the experiments a 30 min adaptation period was allowed before taking the first sample for insulin assay. A second sample was taken 15 min later. These two control samples allowed the assessment of the secretion of the organs in the presence of glucose alone. The physiological medium supplemented with the nucleotide was perfused for 30 min. Insulin was assayed in the efferent fluid from the pancreas using the radioimmunological method B of Hales & Randle (1963). The standard used was pure rat insulin the biological potency of which, was 20.7 iu/mg. The anti-insulin antibody was the antibody INSIK-1 from C.E.A. (Commissariat à l'Energie Atomique). (Cross-reactions: Proinsulin 7%, C peptide $<<$ 0.01%). In the method of assay the coefficient of variation intra-assay was 9% and inter-assay: 13.5%.

The kinetics of insulin output rate were studied for each nucleotide analogue and for each dose. The results for each point were calculated as a percentage of the starting value just before adding the adenine nucleotide analogue: the values obtained were 'normalized insulin output rate'. Samples were taken every min during the first 5 min, then at time 7, 10, 15, 20 and 30 min. The areas under the curves were calculated for each experiment during the 30 min when the nucleotide was added to the perfusion medium, using the normalized insulin output rate.

We investigated whether the response was dose-related. To do this we determined the mean normalized insulin output rate applying the following ratio:

$$\frac{\text{Area under curve}}{30}$$

The mean normalized insulin output rate was plotted as a function of the logarithm of nucleotide concentration. Three concentrations were studied for each analogue: 1.65, 16.5 and 165 μmol/l. These were selected because they were located on the linear part of the dose-response curve obtained with ATP (Loubatières-Mariani *et al.*, 1979). In one case a lower concentration was studied.

β,γ-Methylene ATP, α,β-methylene ATP and α,β-methylene ADP were obtained from SIGMA Chemical Company and were in the acid free form.

Results

Effect of β,γ-methylene ATP

The results obtained with β,γ-methylene ATP are shown in Figure 2. The secretion of insulin was not stimulated by β,γ-methylene ATP at the three concentrations used: 1.65, 16.5 and 165 μmol/l. A mild but significant ($P<0.01$) inhibition of insulin release was evident with the concentration of 16.5 μmol/l. The time course of insulin secretion from control pancreas perfused only with a perfusion medium containing glucose 8.33 mmol/l is plotted in Figure 2. The insulin output rate at time 45 min was: 32.3 ± 7.3 ng/min (mean ± s.e. mean, n = 9).

Effect of α,β-methylene ATP and α,β-methylene ADP

α,β-Methylene ATP and α,β-methylene ADP added to the perfusion medium stimulated insulin secretion (Figures 3 and 4).

The stimulation induced by α,β-methylene ATP was biphasic as with ATP. However, the first peak was slightly delayed; there was a brief inhibition during the first min, because a decrease in flow rate induced by this drug biased the first response of insulin release (Figure 3). The response to α,β-methylene ATP became manifest at 1.65 μmol/l; from this concentration and up to 165 μmol/l there was a linear relationship between the increase of insulin output and the logarithm of the concentration. Thus, α,β-methylene ATP stimulated insulin release in a dose-dependent manner (Figure 5). The calculated regression line is shown in Table 1.

The results obtained with α,β-methylene ADP on insulin release showed a pattern identical to ATP (Loubatières-Mariani *et al.*, 1979). The stimulation was biphasic. At the three concentrations used (1.65, 16.5 and 165 μmol/l) there appeared an immediate first phase which culminated at the 2nd min. Unlike the effects observed with α,β-methylene ATP, this first phase was not delayed because α,β-methylene ADP had no effect on the flow rate of the pre-

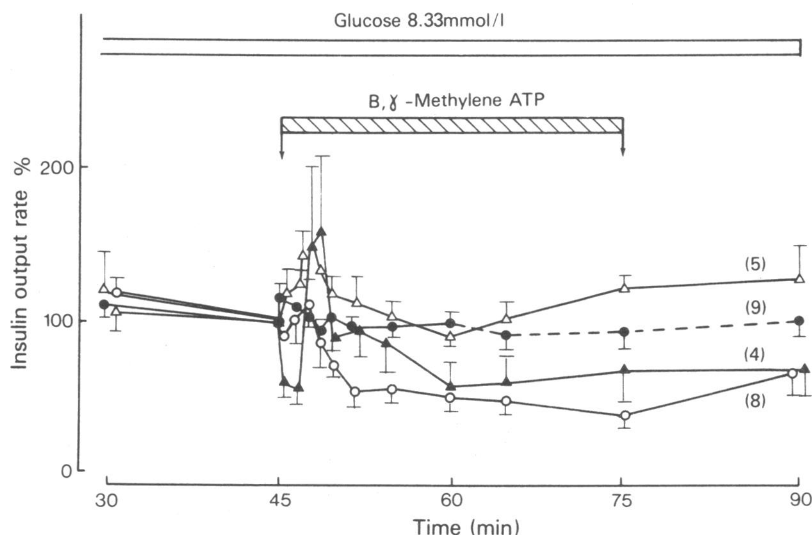

Figure 2 Effects of various concentrations of β,γ-methylene ATP on insulin secretion from the isolated perfused pancreas of the rat: (▲) 165 μmol/l; (○) 16.5 μmol/l; (△) 1.65 μmol/l; β,γ-methylene ATP; (●) controls. Each point represents the mean of the number of experiments indicated in parentheses; vertical lines show s.e. mean.

paration. The response to α,β-methylene ADP was related to the concentration. The calculated regression line is shown in Table 1.

Relative potency of methylene analogues of ATP and ADP in comparison with ATP

A comparison of potency was performed by the parallel line assay method (Armitage, 1980), between the stimulatory nucleotide analogues studied in this paper and ATP, previously taken as a reference agent (Loubatières-Mariani *et al.*, 1979). We carried out a linear regression analysis from the values of mean normalized insulin output rate (cf. methods). The three dose-response curves did not deviate significantly from parallelism. The results are shown in Table 1. ATP was 1.2 fold more potent than α,β-methylene ATP; in contrast α,β-methylene ADP was 3.2 fold more potent than ATP.

Discussion

In our experiments, among methylene analogues of ATP, α,β-methylene ATP proved to be an agonist with a similar activity to that of ATP, whereas β,γ-methylene ATP had no stimulatory effect on insulin secretion.

The advantage of the methylene analogues of ATP and ADP is the extreme stability of the P-C-P bonds which cannot undergo enzymatic cleavage. On the other hand, the structural characteristics (P-X bond length and P-X-P bond angle) of the P-C-P grouping are substantially different from the P-O-P grouping; furthermore the ionisation constant of the last diphosphonate hydrogen of β,γ-methylene ATP is much smaller than the comparable hydrogen for ATP, thus the net charge of β,γ-methylene ATP may be quite different from ATP at pH 7 (Yount, Babcock, Ballantyne & Ojala, 1971a; Yount, 1975). We have previously shown that another analogue of ATP, AMP-PNP, is an agonist which is about ten fold less potent than ATP (Loubatières-Mariani *et al.*, 1979). P-N-P grouping is remarkably similar to P-O-P grouping, thus AMP-PNP should mimic ATP more effectively (Yount *et al.*, 1971a). If we consider the compounds β,γ-methylene ATP, AMP-PNP and ATP, we can also observe that the bridging atom i.e. CH_2, NH and O becomes more and more electronegative. With myosin systems, AMP-PNP is effective in replacing ATP, which is not the case with β,γ-methylene ATP (Moos, Alpert & Myers, 1960; Yount, Ojala & Babcock, 1971b). In the same way AMP-PNP has an agonist activity on insulin release and β,γ-methylene ATP does not.

If β,γ-methylene ATP is not a substrate for enzymes, which cleave the β,γ pyrophosphate linkage of ATP, it seems to be an eventual substrate for enzymes which cleave the α,β-linkage. A calcium ion-dependent adenosine triphosphate pyrophosphohydrolase, which hydrolyzed 5′-adenyl-methylene-diphosphonate to AMP, was identified in rat liver plasma membranes and found to purify with 5′-nucleotidase (Flodgaard & Torp-Pedersen, 1978). Phillis, Kostopoulos, Edstrom & Ellis (1979) found that the β,γ-methylene analogue of ATP and AMP-

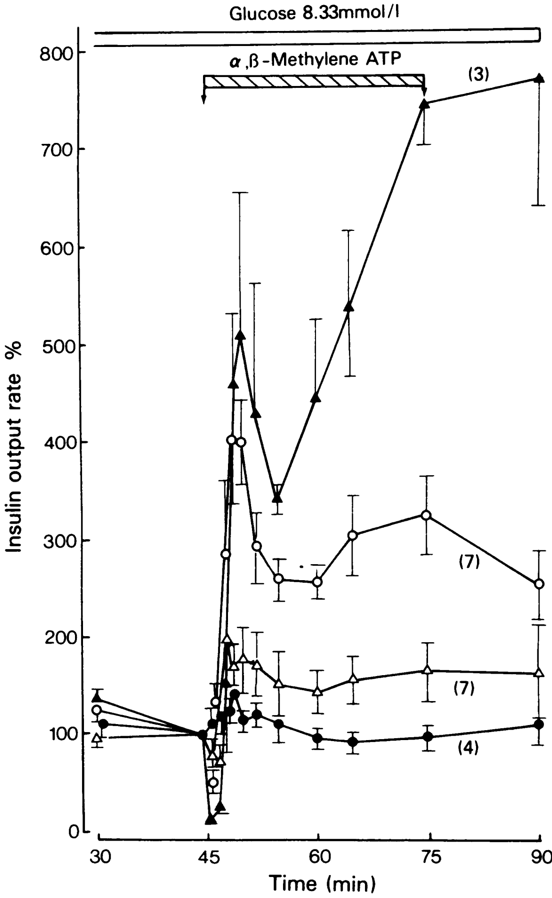

Figure 3 Effects of various concentrations of α,β-methylene ATP on insulin secretion from the isolated perfused pancreas of the rat: (▲) 165 μmol/l; (○) 16.5 μmol/l; (△) 1.65 μmol/l; (●) 0.165 μmol/l α,β-methylene ATP. Each point represents the mean of the number of experiments indicated in parentheses; vertical lines show s.e. mean.

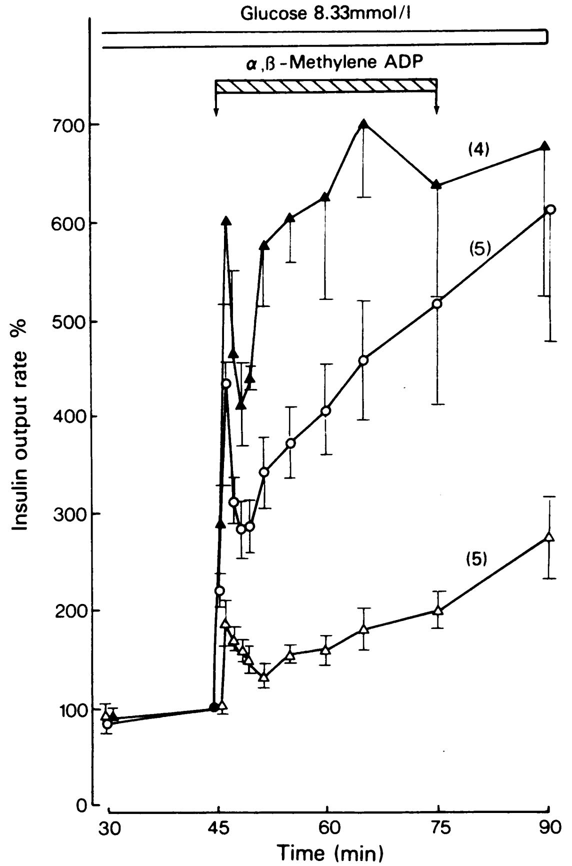

Figure 4 Effects of various concentrations of α,β-methylene ADP on insulin secretion from the isolated perfused pancreas of the rat: (▲) 165 μmol/l; (○) 16.5 μmol/l; (△) 1.65 μmol/l α,β-methylene ADP. Each point represents the mean of the number of experiments indicated in parentheses; vertical lines show s.e. mean.

PNP possessed a quite pronounced depressant activity on cerebral cortical neurones, whereas the α,β-methylene analogues of ADP and ATP had only weak activities in this respect. These authors explain their results by the fact that a prior degradation of the adenine nucleotide to adenosine is necessary before the effect can occur on the central neurones and activate the adenosine receptor. In contrast, the insulin releasing response elicited by the methylene analogues of ATP does not depend upon the conversion to AMP and adenosine. Indeed β,γ-methylene ATP which may be hydrolyzed to AMP and adenosine was ineffective. This result is in accordance with previous findings since AMP and adenosine had only a very weak insulin releasing activity (Loubatières-Mariani *et al.*, 1979). On the other hand the structural characteristics and electronic charge between the 2nd and 3rd phophate seem very important for the binding with the receptor to elicit an insulin secretory effect.

If we consider the two structural analogues with methylene residue between α and β phosphorus atoms (α,β-methylene ATP and α,β-methylene ADP) we can observe that they are as potent or more potent than ATP. Thus the same structural change which suppresses the activity when it is located on one site (between β and γ phosphorus of ATP) does not modify the activity when it is located on another site (between α and β phosphorus). Consequently the structural features between the first 2 phosphorus

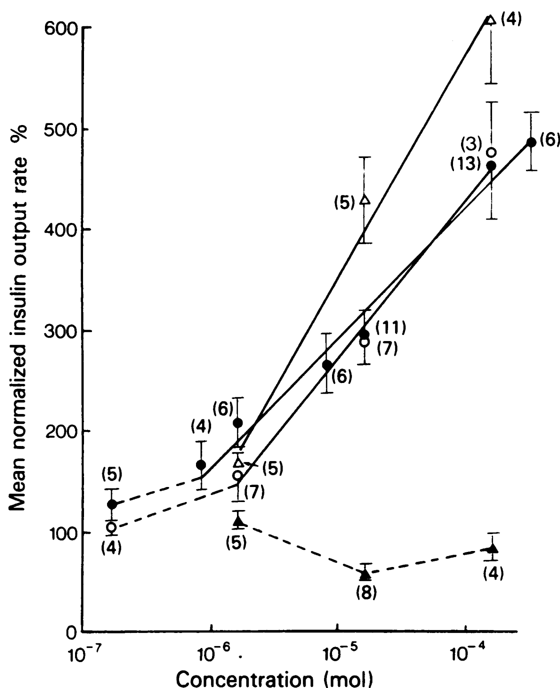

Figure 5 Dose-response curves of ATP (●), α,β-methylene ATP (○) β,γ-methylene ATP (▲) and α,β-methylene ADP (△) on insulin secretion. Solid lines represent the calculated regression lines. Each point represents the mean from the number of experiments indicated in parentheses; vertical lines show s.e. mean.

atoms do not seem to play a prominent part in the binding with the receptor. On the contrary since ADP, α,β-methylene ADP, ATP and α,β-methylene ATP have an agonist activity, while β,γ-methylene ATP has not, the linkage P-O- at the level of the 2nd phosphorus seems to be of importance for the binding with the receptor.

The observations made in this study substantiate our previous results indicating that the presence of di- or triphosphate groups are essential for the insulin secretory action of adenine nucleotides. Comparable conclusions were advanced, from experiments on the guinea-pig taenia coli. In this preparation Satchell & Maguire (1975) found that optimal inhibitory potency in adenine nucleotide derivatives is associated with the presence of di- and triphosphate groups.

Our findings provide support for the purine receptor hypothesis of Burnstock (1976). Recently Burnstock (1978) suggested two such receptors may exist: P_1 being relatively specific for adenosine and P_2 for ATP. The latter group appears to be predominant in the gastrointestinal tract and urogenital system. The present data suggest that a receptor for ATP may be located on the insulin secretory cells and emphasize the importance of the steric and electronic characteristics of the polyphosphate chain for the binding with this hypothetical receptor.

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Table 1 Regression lines and relative potency of methylene analogues of ATP and ADP

	Regression lines	*Relative potency* (95% confidence limits are given in parentheses)
ATP	y = 128 x + 160	
α,β-methylene ATP	y = 154 x + 114	ATP: α,β-methylene ATP = 1.2 (0.5 − 3.0)
α,β-methylene ADP	y = 218 x + 128	ATP: α,β-methylene ADP = 0.31 (0.11 − 0.89)

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